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ORIGINAL RESEARCH—BASIC SCIENCE

Intracavernosal Basic Fibroblast Growth Factor Improves Vasoreactivity in the Hypercholesterolemic Rabbit

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ABSTRACT

Purpose. We determined the effects of intracavernosal injection (ICI) of recombinant basic fibroblast growth factor (rbFGF) on corporal tissue in hypercholesterolemic rabbits.

Methods. Twenty New Zealand White rabbits were fed a 1% cholesterol diet for 6 weeks and were randomly divided into four groups. Group 1 (N = 5) received an ICI of phosphate buffered saline solution (PBS) once and again 3 weeks later. Group 2 (N = 4) received an ICI of 2.5 µg rbFGF once and PBS 3 weeks later. Group 3 (N = 6) received an ICI of 2.5 µg rbFGF once and again 3 weeks later. Group 4 (N = 5) received an ICI of 2.5 µg rbFGF once. All animals were maintained on the high cholesterol diet until sacrifice, 3 weeks after last injection. Strips of corporal tissue were submaximally contracted with norepinephrine, and dose–response curves were generated to evaluate endothelial-dependent (acetylcholine, ACH) and endothelial-independent (sodium nitroprusside, SNP) vasoreactivity. Protein levels of bFGF and vascular endothelial growth factor (VEGF) were assessed by enzyme-linked immunosorbent assay. Neuronal nitric oxide synthase (nNOS) protein and mRNA were detected by Western blot and semi-quantitative polymerase chain reaction, respectively.

Results. Vasoreactivity was improved by bFGF treatment as shown by higher ED50[−log(M)] of ACH and SNP in Groups 2, 3, and 4. The expression of bFGF protein, VEGF protein, nNOS protein, and mRNA were all increased after bFGF treatment.

Conclusions. ICI of bFGF improved vasoreactivity in hypercholesterolemic rabbit corporal tissue, offering a new direction to explore for the treatment of erectile dysfunction. Xie D, Pippen AM, Odronic SI, Annex BH, and Donatucci CF. Intracavernosal basic fibroblast growth factor improves vasoreactivity in the hypercholesterolemic rabbit. J Sex Med 2006;3:223–232.

Key Words. Animal Models; Vascular Physiologic Studies of Genital Arousal; Pharmacologic Studies in Sexual Function; Penis; Impotence; Angiogenic Growth Factors; Fibrosis; Endothelium

Introduction

Erectile dysfunction (ED) afflicts approximately 20–30 million men in the United States [1]. Normal penile erection is predominantly a vascular event that involves interaction between endothelial and smooth muscle cells in the corpus cavernosum [1,2]. The principal event in normal penile erection is trabecular smooth muscle relaxation mediated by the release of neurotransmitters from cavernous nerve terminals and nitric oxide from endothelial cells [2,3]. Although ED may result from psychological, neurological, or arterial ischemic causes, the major etiology of ED is structural alterations of the cavernous smooth muscle and endothelium and a resulting decrease in vasomotor reactivity [1].

Hypercholesterolemia is a major risk factor for the development of ED. In men, every millimole per liter increase in total cholesterol results in a 1.32-fold increase in the risk of ED [4]. The hypercholesterolemic rabbit model of ED is an established model of nontraumatic vascular injury that results in detrimental structural changes in corporal tissue. The vascular injury begins with a breakdown of endothelial integrity which occurs without gross structural changes [5]. As the amount of injury increases, qualitative and quantitative abnormalities become evident in the number and function of vascular smooth muscle cells that are similar to the reductions seen in humans with ED [5–9].

Angiogenesis is the growth and proliferation of blood vessels from existing vascular structures [10]. A number of angiogenic growth factors, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), are known to be present in vascular structures [10–14]. In the setting of vascular injury, angiogenic growth factors can act as survival factors for the microvascular endothelium [10,13,14]. VEGF has beneficial effects on the structure and function of corporal smooth muscle cells in hypercholesterolemic rabbits. Additionally, the effect of systemic (intravenous) VEGF on corporal tissue was superior to the effect of local intracavernosal injected (ICI) VEGF on corporal tissue [14]. bFGF is an 18 kDa protein with a strong affinity for heparin sulfate molecules on the cell surface and in the extra cellular matrix and bFGF binds to a family of receptors found on multiple cell types [12]. In a preclinical model of hypercholesterolemic arterial injury, intravenous bFGF was able to protect arterial endothelium [13]. However, bFGF also has the potential to cause fibrosis, and systemic bFGF has been shown to cause drug-related toxicities, including proteinuria and hypotension [15–17]. In a prior study, we found that systemic bFGF induces favorable histological changes in the corpus cavernosum of hypercholesterolemic rabbits [18]. Therefore, the goal of the current effort was to explore the effects of ICI of bFGF on erectile corporal tissue in the same hypercholesterolemic rabbit model.

Materials and Methods

Animal Model

A total of 20 male New Zealand White (NZW) rabbits weighing 2 to 3 kg (14 weeks old) were fed a 1% cholesterol diet (Harland Teklab, Madison,

WI) continuously for the duration of this study. After 6 weeks, the rabbits were randomly divided into four experimental groups. Group 1 (N = 5) received an ICI of placebo (phosphate buffered saline solution, PBS) at day 1 and again 3 weeks later. Group 2 (N = 4) received an ICI of 2.5 µg recombinant bFGF (rbFGF) in PBS at day 1 and PBS 3 weeks later. Group 3 (N = 6) received an ICI of 2.5 µg rbFGF at day 1 and again 3 weeks later. Group 4 (N = 5) received an ICI of 2.5 µg rbFGF at day 1 and no subsequent injection. All rabbits were sacrificed 3 weeks after the last injection. All protocols and procedures involving animals conformed to the Guidelines for Use of Laboratory Animals from the United States Department of Health and Human Services, and they were approved by the Duke University Animal Care and Use Committee. All animals received care in accordance with Principles of Laboratory Animal Care from the National Society for Medical Research and Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (NIH), Publication no. 86–23, revised 1985.

Tissue Procurement, Histological Section Preparation, Protein and mRNA Isolation

As described previously, at the end of the study all rabbits were anesthetized with ketamine and xylazine, and penectomy was performed with careful dissection of the corpora cavernosa from the tunica albuginea before the rabbits were sacrificed [18].

The middle portion of the corporal tissue was placed in 30% sucrose-PBS and embedded in cross section in Tissue-Tek optimal cutting temperature compound (Sakura, Torrance, CA). The remainder of the corporal tissue was snap-frozen in liquid N_2 for protein extraction and RNA isolation. Cryostat sections (5 µm) were prepared on Superfrost Plus (Fisher Scientific, Pittsburgh, PA) microscope slides for histological analysis.

For protein analysis, tissue samples were pulverized in liquid N_2 and homogenized in 3 to 5 × volumes 10 mM tris (hydroxymethyl) aminomethane (Tris), pH 7.4, and 100 mM NaCl using a Polytron (Brinkmann, Westbury, NY). The suspension was centrifuged twice at 8,000 × g at 4°C for 15 minutes, and the protein content of the supernatant was determined by Bradford assay. Total RNA was islolated with Trizol (Gibco BRL, Life Technologies, Inc., Grand Island, NY) reagent according to manufacturer instructions

with a slightly modified method. Briefly, tissue samples were homogenized in 1 mL Trizol, incubated for 10 minutes at room temperature, and 200 μL chloroform was added. After centrifugation at $13,000 \times g$ for 20 minutes at 4°C, the aqueous phase was collected and RNA was precipitated and washed with isopropyl alcohol and 75% ethanol sequentially. RNA concentration was determined by spectrophotometry.

Vasoreactivity Studies

For isometric tension studies, two corporal strips (0.8 cm length each) from each animal were suspended in 15 mL capacity organ baths containing Krebs physiological salt solution (122 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgCl2, 2.5 mmol/L CaCl2, 15.4 mmol/L NaHCO3, 1.2 mmol/L KH2PO4, and 5.5 mmol/L glucose) maintained at 37°C and oxygenated with 95% O2 and 5% CO2. After equilibration at 0.5 g, optimal preload tension was determined by contracting strips with 60 mmol/L KCl Krebs solution (60 mmol/L NaCl, 1.2 mmol/L MgCl2, 2.5 mmol/L CaCl2, 15.4 mmol/L NaHCO3, 1.2 mmol/L KH2PO4, and 5.5 mmol/L glucose) at incrementally increasing levels of preload until a further increase in tension failed to generate an increase in active tension (total tension minus resting tension) of at least 10%. All subsequent testing was then performed at the optimal resting tension for each strip. Strips were submaximally precontracted with 10^{-5} M norepinephrine, and after a contractile plateau was reached 10^{-8} to 10^{-3} M acetylcholine (ACH) or 10^{-8} to 10^{-4} M sodium nitroprusside (SNP) was added cumulatively in logarithmic increments. Endothelial-dependent relaxation was assessed using ACH, while endothelial-independent relaxation was assessed by SNP. Relaxation in response to each dose of ACH or SNP is expressed as a percent of the active tension generated by the 10^{-5} M dose of norepinephrine. These values were plotted against the negative logarithm of the agonist dose to produce relaxation dose–response curves. Logistic regression analysis with log transformation was performed on the cumulative dose–response curves of each treatment group to determine the ED50 of each agent. Groups were compared at the ED50 of ACH and SNP [19].

Histological Evaluation of Smooth Muscle, Endothelial, Cell, and Collagen Content

To assess the content of vascular smooth muscles and endothelial cells in the corporal tissue, immu-

nohistochemistry was performed using previously described antibodies and conditions [14,19]. Briefly, frozen sections were allowed to reach room temperature, and placed in ice-cold acetone for 10 minutes, and then washed in PBS three times, 5 minutes each. Blocking solution (10% normal horse serum in PBS) was applied for 20 minutes at room temperature. Mouse monoclonal anti-human muscle actin HHF35 antibody (Dako Corp., Carpinteria, CA) (1:500) and mouse monoclonal anti-human endothelial CD31 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (1:20) diluted in PBS were applied overnight at 4°C. Sequential incubations with biotinylated anti-mouse IgG and avidin-biotin-peroxidase complex reagent using a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) were performed according to manufacturer instructions. Levamisole was added to block endogenous alkaline phosphatase activity. Immune complexes were localized using alkaline phosphatase substrate Vector Red (Vector Laboratories, Inc., Burlingame, CA). Sections were counterstained with hematoxylin, dehydrated, and mounted with Permount (Fisher Scientific, Pittsburgh, PA). The positive signal was red. Human vessels served as the positive control and PBS was used instead of the primary antibody as the negative control. Collagen content in corporal tissue was measured by Sigma Accustain (Sigma Diagnostics, St. Louis, MO) Masson's trichrome staining according to manufacturer instructions. Collagen appears as a blue color.

For analysis, six randomly selected fields per sample at $200 \times$ magnification were acquired with a 1×70 microscope (Olympus Optical Corp., Melville, NY) and Premiere 5.0 (Adobe, San Jose, CA) software. Images were analyzed using NIH 1.62 image analysis software (NIH, Bethesda, MD). The overall smooth muscle (actin), endothelial cell (CD31), and collagen (blue) area was quantified by the percent positive area per 200X field calculated. The reader, blinded to sample type, performed all analyses.

Measurement of bFGF Protein, VEGF Protein

Protein concentrations of bFGF and VEGF in corporal tissue protein lysates were determined using a solid-state enzyme-linked immunoassay (ELISA) system with a Quantikine bFGF and VEGF ELISA Kit (R & D Systems, Minneapolis, MN), as described previously [18]. Assay sensitivity was 5 pg/mL for recombinant VEGF and 10 pg/mL for recombinant bFGF and results were

Table 1 The primary and secondary antibodies

Antibodies	Source	Concentration	Incubation time
eNOS	Mouse Monoclonal antibody IgG1 (BD Transduction Laboratories).	1:1,000	Overnight at 4°C
p-eNOS	Polyclonal antibody against the phosphorylated Ser1177 of eNOS (Cell Signaling)	1:2,000	Overnight at 4°C
nNOS	Mouse Monoclonal antibody IgG2a (BD Transduction Laboratories).	1:1,000	Overnight at 4°C
Actin	Mouse Monoclonal antibody IgG (Sigma)	1:3,000	60 minutes at room temperature
Goat antimouse IgG-HRP	Santa Cruz Biotechnology, Inc.	1:10,000	30 minutes at room temperature
Goat antirabbit IgG-HRP	Santa Cruz Biotechnology, Inc.	1:10,000	30 minutes at room temperature

expressed in pg/mL based on standard recombinant protein curves [19]. The assay was validated for up to 100 μg total protein per well.

Determination of NOS by Western Blotting

Western blot analysis for endothelial nitric oxide synthase (eNOS), phosphorylated eNOS (p-eNOS), and neuronal NOS (nNOS) expression was performed using methods described previously [20, 21]. A total of 25 μg of protein per sample was separated on a 4–20% polyacrylamide gel and electroblotted on nitrocellulose membranes. The membrane was blocked, probed with primary antibodies at 4°C overnight, washed, and incubated with secondary antibody. The antigen–antibody complexes were visualized with enhanced luminescence reagent (Amersham Biosciences, Buckinghamshire, England), followed by exposure to Kodak XAR-5 film. Actin was used to control for protein loading. The same blot was stripped and reprobed using mouse anti-human actin antibody and a goat anti-mouse IgG secondary antibody. The intensity for each band was quantified by NIH image software. The working concentrations and conditions are shown in Table 1.

Determination of NOS by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Semi-quantitative RT-PCR was performed using 1 μg total RNA for first strand cDNA synthesis.

RNA was reverse transcribed for 12 minutes at 42°C using Multi-Scribe (AB Applied Biosystems, Foster City, CA) RT and random hexamers. The products were amplified using Aplitic Gold DNA Polymerase with a Gene Amp Gold RNA PCR Reagent Kit (AB Applied Biosystems) according to manufacturer instructions. The primer sequences and PCR conditions are in Table 2. As a control, cDNA for glyceraldehyde 3-phosphate dehydrogenase was amplified. PCR products were analyzed by 1.5% agarose gel electrophoresis [22–24].

Statistical Analysis

Unless otherwise stated, results are expressed as the mean ± SD. Statistical significance was evaluated by Student's t-test for paired or unpaired variables, with $P < 0.05$ considered statistically significant.

Results

Although ICI of VEGF was not to be very effective in this rabbit model of ED [14], isometric tension studies demonstrated that ICI of rbFGF did improve vasoreactivity. Endothelial-dependent vasoreactivity was improved by rbFGF treatment as shown by higher ED50[–log (M)] of ACH and SNP in Groups 2, 3, and 4. The ED50[–log (M)] to ACH was 3.63 ± 0.44 in Group 1, 4.45 ± 0.53 in Group 2 ($P = 0.03$ vs. Group 1), 4.69 ± 0.46 in Group 3 ($P = 0.02$ vs. Group 1), and

Table 2 Nitric oxide synthase (NOS) isoforms oligonucleotides were used for semi-quantitative reverse transcriptase-polymerase chain reaction

| Target gene	Sequence (5'→3')	Annealing tem., °C	No. of cycle	Product size (bp)	Reference
eNOS	CAGTGTCCAACATGCTGCTGGAAATTG(S) TAAAGGTCTTCTTCCTGGTGATGCC (AS)	50	30	485	[22]
nNOS	TGTGTGGGCAGGATCCAGTG(S) GGGACAGGCGCTGAACTCCA(AS)	58	35	509	[23]
GAPDH	GCGCCTGGTCACCAGGGCTGCTT(S) TGCCGAAGTGGTCGTGGATGACCT(AS)	63	27	465	[24]

eNOS = endothelial nitric oxide synthase; nNOS = neuronal nitric oxide synthase; GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

4.72 ± 0.16 in Group 4 (P < 0.01 vs. Group 1). There was no significant difference among Groups 2, 3, and 4. The response to SNP was 5.59 ± 0.16 in Group 1, 5.81 ± 0.12 in Group 2 (P = NS vs. Group 1), 5.81 ± 0.28 in Group 3 (P = NS vs. Group 1), and 5.86 ± 0.11 in Group 4 (P = 0.02 vs. Group 1). There was no significant difference among Groups 2, 3, and 4. Figure 1A and B show the results of the vasoreactivity studies.

As demonstrated by ELISA, ICI of bFGF was associated with higher corporal tissue level of bFGF protein by ELISA (Figure 2A) in Groups 2, 3, and 4 (358.51 ± 30.41, 465.43 ± 89.06, and 955.19 ± 126.1 pg/mL, respectively, with all

P < 0.01) vs. Group 1 (251.11 ± 44.39 pg/mL). The differences were also statistically significant between Group 4 vs. Group 2 or Group 3 (each P < 0.01). We found previously that systemic bFGF led to increases in corporal VEGF [18]. Similarly, in this current study, ICI of bFGF was associated with higher corporal tissue levels of VEGF (Figure 2B) in Groups 2, 3, and 4 (1,381.9 ± 257.3, 2,561.3 ± 317.2, and 2,119.3 ± 601.2 with P = < 0.05, P < 0.001, and P < 0.02, respectively) vs. Group 1 (1,006.5 ± 115.7 pg/mL).

Animals treated with ICI of bFGF showed greater corporal endothelial cell and smooth muscle cell content vs. control treated rabbits

Relaxation to Acetylcholine

A

Relaxation to Sodium Nitroprusside

B
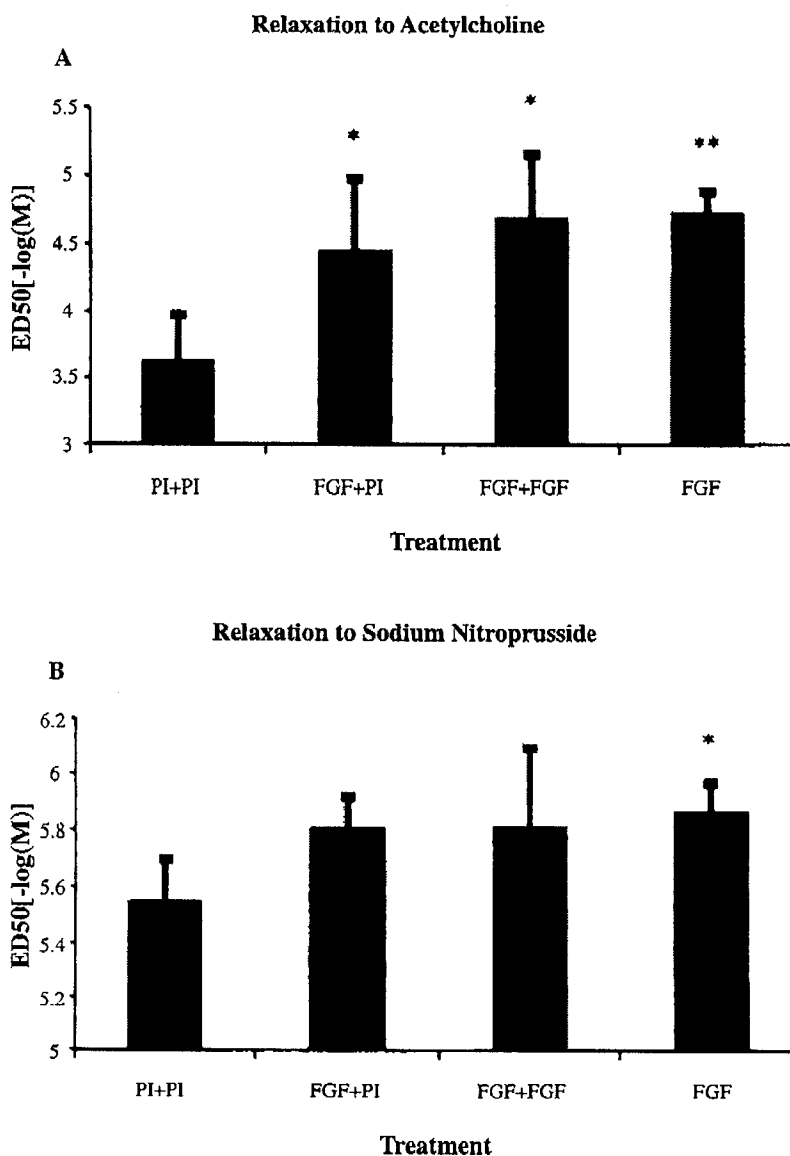
Figure 1 Comparison of effective doses (ED50) of acetylcholine (ACH) and sodium nitroprusside (SNP) to gauge relaxation response of corporal tissues in isometric tension analysis with or without intracavernosally basic fibroblast growth factor (bFGF) injection. bFGF treatment did lead to the improvement in vasoreactivity. Endothelial-dependent and independent reactivity were improved by bFGF treatment as shown by higher ED50[−log(M)] of ACH and SNP in Groups 2, 3, and 4. *P < 0.05, **P < 0.01, ***P < 0.001 vs. Group1. PI = placebo, FGF = bFGF.
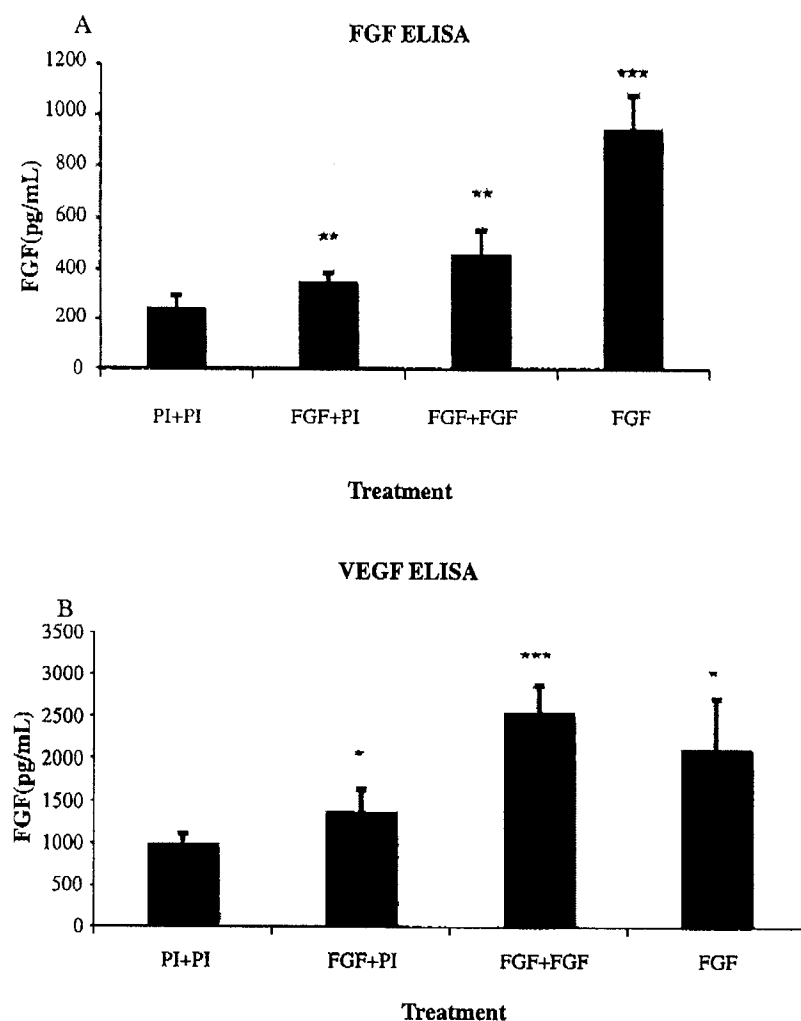
Figure 2 On enzyme-linked immunoassay (ELISA) basic fibroblast growth factor (bFGF) protein in corporal tissue was lower in Group 1 than in Group 2 or 3 and 4 (each $P < 0.01$). On ELISA vascular endothelial growth factor (VEGF) protein in corporal tissue was lower in Group 1 than in Group 2 or 3 and 4 (each $P < 0.01$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Group1. PI = placebo, FGF = bFGF.

(Figure 3A–D). The content of endothelial cells in corporal tissue in Group 1 was $0.658 \pm 0.03\%$, which was significantly lower than in Group 2 ($0.725\% \pm 0.03$, $P < 0.05$ vs. Group 1), Group 3 ($0.743\% \pm 0.03\%$, $P < 0.05$ vs. Group 1) and Group 4 (0.753 ± 0.05, $P < 0.05$ vs. Group 1). The fraction of smooth muscle cells in corporal tissue in Group 1 was $20.36\% \pm 1.39\%$, which was significantly lower than in Group 2 ($26.61\% \pm 1.71\%$, $P < 0.01$ vs. Group 1), Group 3 ($34.15\% \pm 2.65\%$, $P < 0.001$ vs. Group 1), and Group 4 ($32.63\% \pm 2.94\%$, $P < 0.01$ vs. Group 1). The fraction of collagen content (Figure 3E and F) in corporal tissue was not significantly different among Groups 1 to 4 ($1.37\% \pm 0.55\%$, $1.48 \pm 0.39\%$, $1.77\% \pm 0.24\%$, and $1.43 \pm 0.37\%$, respectively).

Western blot analysis of eNOS (140KD) protein demonstrated no difference in eNOS protein

among the groups (Figure 4A). Similarly, there was no significant change in the eNOS mRNA level. However, p-eNOS (140KD) was increased 3.0, 3.5, 3.6 times in Group 2, 3, and 4 relative to Group 1 (Figure 4A), and nNOS protein (160KD) was increased 2.5, 3.2, 3 times in Group 2, 3, and 4 relative to Group 1 (Figure 4A). The increase in nNOS protein in corporal tissue correlated with an increase in nNOS mRNA, which was obtained from semi-quantative PCR (Figure 4B).

Discussion

Initially established in 1991 and further characterized in our laboratory, the hypercholesterolemic rabbit model of ED is useful for studying corporal endothelial and smooth muscle cells' structure and function [14,18,20,25,26]. In our previous studies we found that significant elevation of total
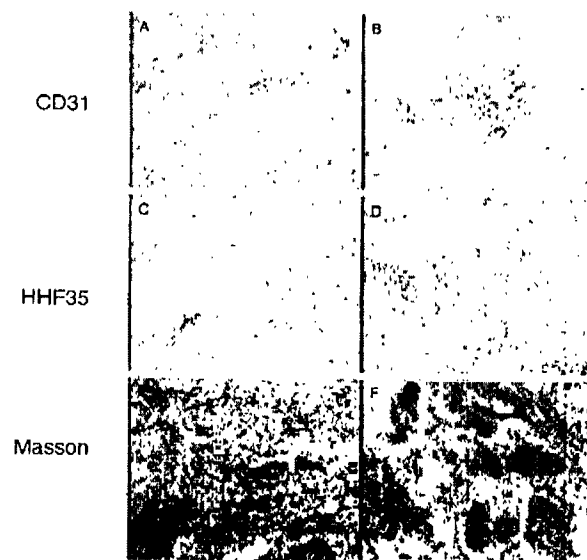
Figure 3 Representative corporal endothelial cell content measured by immunohistochemistry in Groups 1 (A) and 3 (B). Mouse anti-human CD31 antibody. Representative corporal smooth muscle content measured by immunohistochemistry in Groups 1 (C) and 3 (D). Mouse anti-human HHF35 muscle actin antibody. Reduced from ×200. Immunohistochemistry staining shows significantly increased corporal endothelial cell and smooth muscle cell content in Group 3 with bFGF × 2 regimen than in Group 1 with PBS × 2 treatment ($P < 0.05$ and $P < 0.001$, respectively). Representative corporal collagen content in Groups 1 (E) and 3 (F). Masson's trichrome stain, reduced from ×200. Corporal collagen content was not significantly increased in Group 3 compared with Group 1 ($P = 0.253$).

cholesterol levels occurred in serum of rabbits receiving the 1% cholesterol diet for 4 weeks and 12 weeks (727 ± 75.6 mg/dL and 1249.5 ± 69.8, respectively) compared with rabbits with normal diet (38.7 ± 5.53 mg/dL) [18,26]. The model demonstrates time-dependent progression from endothelium-dependent (ACH) and then endothelial-independent (SNP) abnormalities in smooth muscle relaxation being between 4 and 7.5 weeks [14,20].

At its core ED involves an abnormal interaction between endothelial and smooth muscle cells in the corpus cavernosum [1]. Hypercholesterolemia induces injury to endothelial and vascular smooth muscle cells, resulting in abnormal function and content in preclinical models that bear resemblance to abnormalities found in human corporal tissues [1,2,5,6,9]. Angiogenic growth factors like VEGF and bFGF are not only are capable of causing endothelial proliferation, but these cytokines also have the ability to modulate favorably vascular injury [10]. Prior studies from

our group and other groups, have shown that intravenous (systemic) VEGF administration can improve corporal smooth muscle function in vitro and in ex vivo models of ED better than local (ICI) delivery [14,27]. Te et al. [11] reported that bFGF is present at high levels in rat corporal tissue. In a previous study of hypercholesterolemic rabbits, we showed that intravenous bFGF
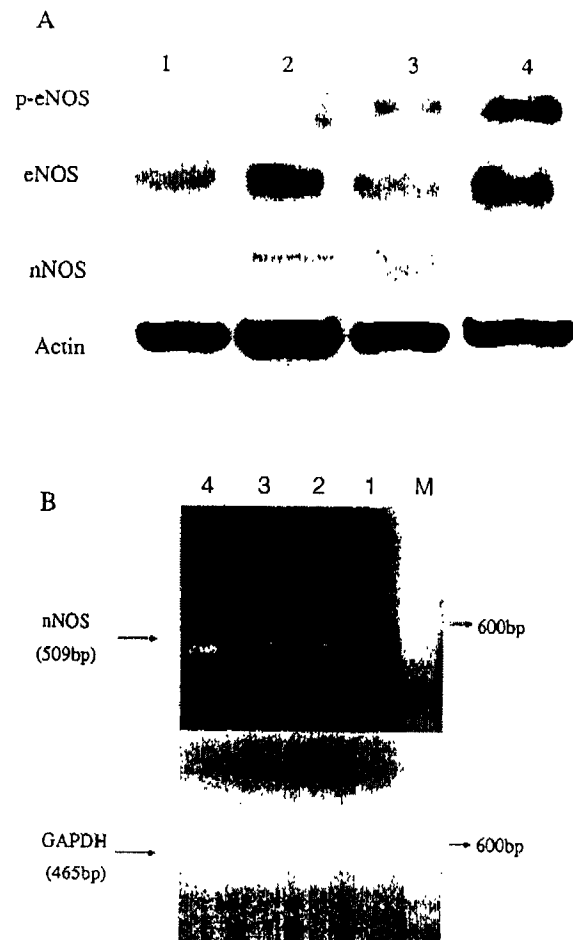
Figure 4 A. In Western blot analysis, there was no difference in endothelial nitric oxide synthase (eNOS) protein following basic fibroblast growth factor (bFGF) treatment; however, phosphorylated eNOS (p-eNOS), neuronal nitric oxide synthase (nNOS) was increased after bFGF treatment. The densitometric analysis revealed a slight increase in p-eNOS and nNOS in Group 3 with bFGF × 2 regimen than in Group 2 with bFGF × 1 treatment. The expressions in Group 3 and Group 4 are almost the same. Groups 1 to 4 corresponding to lanes 1 to 4, respectively. The same blot was stripped and reprobed for actin to demonstrate comparable protein loading. B. On Semi-quantative RT-PCR, there was significantly increased nNOS expression after bFGF treatment, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control. Groups 1 to 4 corresponding to lanes 1 to 4, respectively. M, 100 kb DNA ladder.

showed beneficial effects on corporal tissue histology [18]. However, systemic bFGF is also capable of stimulating smooth cells to secrete collagen and of causing fibrosis in target tissue, as well as of causing proteinurea and hypotension [15–17]. Therefore, this study was conducted to determine if ICI of bFGF had any therapeutic potential in a rabbit model of ED. The major findings of our current study were that the intracavernous administration of bFGF results in an increase in vasoreactivity, endothelial cell content, vascular smooth muscle cell content, bFGF protein, and VEGF protein expression, but no significant increase in corporal tissue fibrosis. Although there were previous reports that bFGF had the potential to cause fibrosis [15], low dosage of bFGF did not increase fibrosis in corporal tissue and plaque formation in aorta [13,18]. In addition, bFGF treatment induced eNOS phosphorylation and nNOS expression. These combined findings could have multiple beneficial effects on dysfunctional corporal tissue, suggesting that bFGF may be valuable in the therapeutic modulation of ED.

While VEGF is specific for endothelial cells, FGFs are also potent growth factors for other cell types such as smooth muscle cells [28]. Both route of administration and the type of angiogenic growth factor used may produce differential effects in other systems [29]. Based on predictions from VEGF, ICI of bFGF would not have been predicted to have much efficacy in this preclinical model of ED. However, we found that ICI bFGF did improve vasoreactivity.

Generation of nitric oxide (NO) represents the final common element that directly stimulates corporal smooth muscle relaxation [1]. The endothelium-dependent pathway requires the production of NO from corporal endothelium in response to acetycholine from nerves. The endothelial-independent pathway produces smooth muscle relaxation from the direct release of NO from efferent nerve terminals. Angiogenic growth factor may have influences on both processes. VEGF leads to the phosporylation (and thus activation) of eNOS and has been shown to mediate vascular endothelial growth factor-induced penile erection [30]. The link between bFGF and eNOS is less direct, although bFGF induced eNOS expression in lymphoma cells [31]. In our current study we demonstrated that bFGF treatment promoted eNOS phosphorylation but had no effect on total eNOS levels. Thus, bFGF and VEGF may act through a similar mechanism (eNOS phosphorylation) to exert beneficial effects on corporal tis-

sue. In corporal tissue, eNOS is expressed in the endothelium and nNOS, which is largely responsible for the production of NO that enters cavernosal smooth muscle cells as part of initial sexual stimulation, and is expressed in the cavernosal nerve [32]. Our experiment also showed that nNOS was induced by bFGF treatment, which may be another important mechanism leading to improvement in vasoreactivity.

In our study, local bFGF treatment significantly increased bFGF and VEGF protein expression in corporal tissue even 3 weeks after the last treatment. Because Group 2 and 3 differed both in total dose and time from last dosing until harvest, a fourth group (Group 4) was used in the this initial study. This group was placed in the study in the anticipation that the results from Group 2 would have been negative, which interestingly, were not. Still when taken together, relative to Group 2, Group 3 had a trend toward a better response to ACH, a trend toward greater endothelial and vascular smooth muscle cell content, and greater corporal tissue content of VEGF. Although the duration of cholesterol feeding differed slightly for Group 4, this group was quite comparable to Group 3. It is interesting to note that Group 4 had significantly higher bFGF protein expression than Group 2 (rbFGF + PBS, $P < 0.01$ each), which probably could have been predicted; however the bFGF values in Group 4 were also greater than in Group 3 (rbFGF + rbFGF), and these would not have been predicted. In addition, in this report we cannot determine if the greater number of cells results in the greater production of cytokines, or if bFGF is directly leading to the induction of VEGF, as reported by other studies and systems [33–35]. Our findings suggest that the induction of bFGF and VEGF after rbFGF treatment may be an important mechanism for the rbFGF effects.

Additional studies will be necessary to examine issues such as optimal dosing and treatment frequency. Suetomi et al. [36] reported the potential use of bFGF to preserve erectile function in a diabetic rat model by incorporating the bFGF into gelatin microspheres for delivery. Using Azan-Mallory staining, they showed a mass decrease in smooth muscle in cavernous tissue in the diabetic group that was not present in the bFGF group. They showed no significant difference in endothelial NOS positive areas and the distribution of the diameter of neuronal NOS positive nerve fibers in cavernous tissue. Although it is still investigational, therapeutic angiogenesis is an exciting

and promising treatment strategy for peripheral artery disease and ischemic heart disease. Our current study adds to other studies of VEGF administration and expands the spectrum of the potential use of angiogenic growth factors to modulate forms of vascular injury, including the treatment of ED.

Conclusions

We have demonstrated previously that systemic but not intracorporal VEGF can restore corporal vasoactive dysfunction in the NZW rabbit hypercholesterolemic model. We now demonstrate that low dosage of bFGF, an angiogenic agent and smooth muscle mitogen, delivered by the intracavernosal route, can also restore endothelial-dependent and independent corporal vasoactive function in this rabbit model. This restoration of vasoactive function may be due to the induction of bFGF protein, VEGF protein, and nNOS activity in hypercholesterolemic rabbit corporal tissue. These effects suggest that bFGF may have clinically significant effects in addressing ED in men.

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Conflict of Interest: None.

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